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AD_____

Award Number: DAMD17-99-1-9139

TITLE: Isolation of Genes Involved in Rac Induced Invasion and Metastasis of Breast Carcinoma Cells

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CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
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REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (1 Jul 99 - 1 Jul 00)

4. TITLE AND SUBTITLE

Isolation of Genes Involved in Rac Induced Invasion and Metastasis of Breast Carcinoma Cells

5. FUNDING NUMBERS

DAMD17-99-1-9139

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Report contains color photos

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13. ABSTRACT ***(Maximum 200 Words)***

A better understanding of the progression of breast cancer to the metastatic state, and of the changes that take place in highly malignant breast cells at a molecular level, is essential to develop more direct forms of screening and therapy. Members of the RhoGTPases, in particular Rac, have recently been implicated in adhesion, motility and invasiveness, processes crucial for progression of breast cancer cells to the metastatic state. We have set out to identify target genes of Rac, as the screen may identify additional diagnostic markers, as well as provide new targets for therapy. To this end, we applied cDNA-RDA and microarray analyses to identify genes which are up- or downregulated as a result of Rac expression. These experiments resulted in the identification of 85 independent gene fragments (among them 23 novel genes), of which 37 were apparently upregulated and 48 downregulated. To ascertain the differential expression of the above genes, we initiated Northern blot analysis. Among the clones reconfirmed for difference in mRNA abundance are previously identified genes associated with tumorigenesis and genes whose predicted protein structure is suggestive for a role in invasion.

14. SUBJECT TERMS

Breast Cancer
Rac invasion/metastasis

15. NUMBER OF PAGES

49

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Van Alst 7/28/2000

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INTRODUCTION

The progression of a breast tumor cell to its metastatic state is generally the major cause of morbidity and mortality of breast cancer patients. The process of primary tumor progression to a metastatic stage is complex and consists of multiple steps involving aberrant functions of the tumor cell, including increased local proteolysis, degradation of extracellular matrix components, invasion, adhesion to and migration through the vascular basement membrane, and proliferation at distant sites (1,2,3,4). Multiple changes, including genetic alterations and, in particular, changes in gene expression resulting in loss of normal cellular regulation, have been anticipated to occur during tumor progression (5,6,7,8). Over the past years, several lines of evidence have demonstrated a role for members of the RhoGTPases, in particular for Rac, in adhesion, invasiveness and metastasis (9,10,11,12,13,14,15,16,17). The first indication of a role for Rac in invasion came from the identification of Tiam1, a guanine nucleotide exchange factor for Rac, in a screen for genes which induce invasion in T lymphoma cells (13). Subsequent studies have shown that constitutive activation of Rac confers an invasive potential to mammary epithelial cells (14), and that Rac is an essential downstream mediator of the $\alpha 6\beta 4$ signaling pathway involved in invasion of breast carcinoma cells (16). We postulated that the identification and characterization of the target molecules involved in Rac1-GTPase-specific effects on invasion and metastasis will provide more insights into the mechanism of tumor progression, as well as possibly identify additional diagnostic markers and new targets for therapy. To this end, we have utilized cDNA-RDA (representational difference analysis of cDNA) in combination with microarray technique to identify genes which are up- or downregulated as a result of Rac expression. As discussed in the following sections, we succeeded in the isolation of several interesting potential genes, whose expression levels are altered as a result of either RacV12 or RacN17 expression.

BODY

Our major goal is to identify target genes of Rac which mediate its effects on invasion and metastasis. Towards this end, we proposed the following: 1) Identify genes that are differentially expressed in epithelial cells which do and do not express RacV12, 2) Analyze and initially characterize the isolated candidate genes, and 3) Determine a role for the candidate gene products in invasion and metastasis. As discussed in detail below, we applied cDNA-RDA in combination with microarray technology, which led to the identification of several interesting potential Rac target genes and started the initial characterization. We have accomplished all the aims we had originally proposed for the first year.

1) Identify genes that are differentially expressed in breast epithelial cells, which do and do not express RacV12.

With an eye on identifying Rac target genes mediating its effects on epithelial cell invasion and metastasis, we performed cDNA-RDA on epithelial cells induced and uninduced for the constitutively active mutant form of Rac, RacV12. In addition, we performed similar cDNA-RDA experiments for RacN17. This mutant form of Rac acts as a dominant negative and interferes with endogenous Rac function (17). In order for cDNA-RDA (or any method developed to identify genes that are expressed at different levels) to be successful, the choice and preparation of the biological sample is important. One has to be able to discern effects that arise

as a result of the experimental change from effects that are artificial. Towards this end, we decided to make use of an inducible expression system to express the Rac mutants. In this case, the samples for comparisons are paired as ideally as possible. They are derived from the same population, and the only perturbations are the presence of inducer and the expression of Rac mutants.

We had originally proposed to establish a T47D cell line expressing constitutively active Rac1V12 under the control of the inducible ecdysone system. However, after extensive analysis, we experienced that this cell line, using the ecdysone system, does not allow for tight expression of RacV12, given expression could be observed in the absence of the inducer, ponasterone A. Hence, we decided to make use of a different breast epithelial cell line, namely Mcf7, and the "Retroviral Tet-Off" system (Clontech) instead of the ecdysone system. As shown for T47D cells, the introduction of RacV12 also confers an invasive potential to Mcf7 cells as well as to MDCK cells, when plated on collagen (18). We opted for the tetracycline system since we had obtained MDCK cells with very tight expression of RacV12 under the control of the tetracycline-off system. This system is based on an artificial promotor consisting of a tetracycline-response element (TRE) coupled to a CMV minimal promotor, which drives expression of a gene of interest in a tetracycline-dependent manner. The transcriptional activity of this cassette harbored by a retroviral backbone and introduced into a host genome depends a) on the presence of the tetracycline receptor whose gene has been previously engineered into the cells' DNA and b) tetracycline (or doxycycline) being absent or present in the culture medium. More specifically, the "Tet-off" system is designed such that medium containing doxycycline activates the tetracycline receptor which secondarily represses the Tet-dependent promotor. Conversely, depletion of doxycycline from the medium causes a conformational change in the tetracycline receptor, resulting in its inactivation and derepression of the target promotor. We obtained an Mcf7 Tet-off cell line from Clontech, which is ready to use as host. This cell line contains the pTet-off regulatory plasmid that expresses the tetracycline-controlled activator (tTA), a chimeric protein composed of the Tet repressor protein fused to the VP16 activator domain. We then infected the Mcf7 Tet-off cells with retroviruses containing the Rac mutants cloned in the pTRE2 response plasmid. The viruses were produced using LinX cells (an amphotropic packaging line provided by Greg Hannon, CSHL) as we previously described (19). To evaluate induction of Rac expression, we performed Western blot analysis under inducible conditions. We succeeded in establishing Mcf7 clones expressing RacV12 and RacN17 under the control of the Tet-off system, and found that 48h in the absence of doxycycline was sufficient to induce expression of RacV12 and RacN17 to a level equal to endogenous Rac. No expression of the Rac mutants was observed in the presence of doxycycline.

cDNA-RDA on RacV12 and RacN17 expressing cell lines.

As previously described in the grant proposal, cDNA-RDA is a modified form of RDA, a PCR based differential cloning method (20,21). In this technique, one DNA population (called the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester. cDNA-RDA uses tester and driver sequences that are derived from isolated mRNA that has been converted into cDNA (22). We performed four sets of cDNA-RDA. In the first two, we used cDNAs isolated

from cells induced for RacV12 and RacN17 expression as tester and cDNAs from uninduced cells as driver, as such to identify genes which are upregulated by RacV12 and RacN17 respectively. In the second two sets, we used cDNAs from RacV12 and RacN17 as driver and cDNAs from uninduced cells as tester to identify genes which are downregulated by RacV12 and RacN17 respectively.

An overview of the steps we took is outlined below and summarized in Figure 1. A detailed protocol to perform cDNA-RDA can be found in our chapter in Methods in Molecular Biology (see appendix). Approximately $5x10^7$ cells of each of the two clones (cells containing RacV12 or RacN17) were grown in the presence of 20 ng/ml doxycycline and each condition was split into two halves. One half of each population was then induced by removal of the doxycycline. mRNA was isolated from cells induced and not induced for RacV12 or RacN17 expression and the isolated mRNAs were subsequently converted into cDNA. After cDNA synthesis, the tester and driver cDNAs were digested with *DpnII*, adaptor-ligated and amplified by PCR using adaptor primers to generate representations (amplicons). Representations from both tester and driver were cleaved with *DpnII* to remove their adaptors, and then tester amplicon only was ligated to a new oligonucleotide adaptor. Subsequently, the tester amplicon was mixed with excess driver amplicon, the two melted and allowed to reanneal during a hybridization step at 67°C for 20hr. Only the target sequences can be exponentially amplified during a subsequent PCR step with tester-adaptor specific primers (Figure 1B). These amplified sequences are called difference products1 (DP1). The iterative hybridization-amplification step was repeated once, at higher stringency, and DP1 was used this time as tester with the same driver as above. The resulting difference products are denoted DP2 (see Figure 1A). To avoid isolating RacV12 or RacN17 as major DP, when using cDNAs from cells expressing RacV12 or RacN17 as tester, we added RacWT cDNA to supplement the drivers in both cases. The resulting difference products (DPs) were then run on a 2% agarose gel. A representative example of our cDNA results is shown in Figure 2A.

As a first standard control to check whether the cDNA-RDA was successful, we performed Southern Blot analyses, using the induced gene, Rac, as the positive control to probe equal amounts of each driver and tester representation, DP1, and DP2, transferred to a nylon membrane. If looking for genes upregulated by Rac, the hybridization signal should be stronger in the tester than in the driver and more stronger in both DP1 and DP2 compared to tester, because of the amplification (see Figure 2B). This will even be the case, although much reduced, when Rac is added back to the driver. We experienced from dot-blot analysis (see below) that adding back Rac reduces the percentage of difference products being Rac by half. If looking for genes downregulated by Rac, the hybridization signal should be stronger in the driver than in the tester and absent from both DP1 and DP2, as can be noted in Fig 2B. After successful completion of the cDNA-RDA experiments, the DP2 products were subjected to further analysis.

2) Analyze and initially characterize the isolated candidate genes.

As can be noted from Figure 2A, a difference product consists of a series of visible bands superimposed on a smear (see also Figure 1C). To retrieve and confirm the differential expression of DP2 sequences in the most efficient way, we made use of microarray technology. The latter was done in collaboration with R. Lucito at CSHL. The DP2 products were digested with DpnII and run on a 2% agarose gel. The whole lane from 800-100bp was cut out, gel

purified, subcloned into a bacterial expression vector, and PCR-amplified. Approximately 400 clones from each library (in total 4 libraries from the 4 cDNA-RDA experiments) were selected at random and their individual inserts were PCR-amplified. The PCR amplified inserts were subjected to microarray analysis.

We employed microarray protocols similar to the ones developed in the laboratory of Pat Brown (23,24). Briefly, in their protocol, PCR-amplified cDNA fragments are arrayed onto a glass slide and used for the comparison of message content of two mRNA samples. The mRNA samples are differentially labeled with the nucleotide derivatized fluorophores Cy3 and Cy5 during preparation of first strand synthesis with reverse transcriptase. The labeled cDNAs are hybridized to the microarray and the fluorescence signal resulting from sequence specific hybridization for each channel is quantitated and compared to each other yielding a ratio, or a measure of the abundance of one message as compared to the other.

In the case of our Rac experiments, the PCR amplified difference products were arrayed on glass slides, and cDNA representations prepared from Rac induced and uninduced cells were differentially labeled and hybridized to the microarrays. A representative example of our microarray experiments is shown in Figure 3. To account for differences in hybridization efficiencies on different parts of the array and for differences in print quality of different spots (referred to hereafter as "features"), we printed in duplicate, using only ratios that are in concordance as measured from replica printings of the same feature. Furthermore, to control for the consequences of unequal efficiency of incorporation of the two different fluorophores for the two sample populations, we apply color reversal. In the latter, the order of the fluorescent labels on a sample pair is reversed, resulting in two sample pairs labeled reciprocally. The two feature ratios obtained from the color reversal are inverted and the four feature ratios from the two experiments are averaged to give a final ratio (24,25,26), allowing us to discern which clones were upregulated or downregulated as a consequence of RacV12 and RacN17 expression. To affirm that the ratios obtained from hybridizations using labeled representations are comparable with ratios obtained when using labeled RNA, we directly labeled mRNA prepared from the RacV12 induced and uninduced cells by using reverse transcriptase and incorporation of Cy3 and Cy5 into first strand synthesis. The labeled RNA was then used for microarray hybridizations of the same format described above. A few examples are given in Table 1 and, as can be noted, a good concordance can be found.

We sequenced all clones found to be differentially expressed based on microarraying (e.g., fluorescence ratio greater than 2). However, before sequencing we performed dot blot analysis to exclude cDNA clones which are identical to Rac. To this end, the PCR-amplified fragments were spotted on a Hybond N+ nylon membrane and the membrane was incubated with radiolabeled Rac probe. At present, 300 clones out of a total of ca. 3,500 clones, derived from four independent cDNA-RDA experiments, were sequenced. We obtained 85 independent gene fragments, of which 23 were novel, and a total of 37 apparently upregulated and 48 downregulated candidate genes. The obtained genes could be categorized into the following groups: genes encoding nuclear, ribosomal, mitochondrial, membrane associated, secreted and cytoskeletal proteins (Schmitz, A., *et al.*, manuscript in preparation). Such a broad spectrum of genes encoding proteins of diverse classes was also previously observed in genome wide transcriptome screens, such as for Ras target genes (27). At present, there are two approaches we are taking to prioritize genes for further analysis. 1) We started to explore the effects of

various drugs known to activate or to interfere with previously characterized downstream signaling pathways of Rac on the transcription of these genes. Numerous growth factors, including PDGF, EGF, and HGF are known to stimulate Rac's activity, and activated Rac has been shown to activate JNK, p38, and ERK MAPK pathways (see Figure 4) (17). We started to compare the transcription levels of the above candidate genes between resting cells and cells stimulated with PDGF, EGF, or HGF. Furthermore, we are setting out to compare the transcription patterns of the candidate genes between cells induced for Rac treated or not treated with selective drugs interfering with JNK, p38 and ERK MAPK pathways. For example, SB202190 and UO126 are shown to specifically interfere with p38 MAPK and MEK (MAPKK) respectively (28,29). All these experiments will be performed by microarraying. 2) To assure that the differences we observed when comparing two representations of cDNAs reflect true differences in mRNA abundance, we initiated northern blot analysis. Thus far, we have examined the expression levels of 8 clones in stable cell lines induced or not induced for RacV12 and RacN17, using [α^{32}P]dCTP-random labeled cDNAs (from the 8 candidate genes) as probes, and reconfirmed their difference in mRNA abundance (data not shown). Only clones which have their altered expression pattern reconfirmed by Northern blotting will be further pursued. *The identities of the 8 clones are described below. CDO and VH16 were clones found to be downregulated by RacV12. CDO is a member of the Ig/fibronectin type III repeat subfamily of transmembrane receptors previously demonstrated to be downregulated by oncogenes, such as RasV12 (30). VH16 is a dual specificity phosphatase shown to dephosphorylate activated MAPK (31). Furthermore, Myosin II, calcyclin, hBMP-4 and two novel clones of which ESTs are available, were found as clones upregulated by RacV12. Calcyclin is a calcium binding protein from the S100 family, found to be upregulated in a variety of primary and metastatic tumors (32,33). hBMP-4 (human bone morphogenetic protein-4) is a member of the transforming growth factor β family (34,35). Finally, a clone with homology to extracellular proteinase inhibitor HE4 was found to be upregulated by RacN17 (36). We were very pleased to see that some of the clones identified have previously been associated with tumorigenesis (e.g. calcyclin) and that the nature of some of the molecules, e.g. HE4, is suggestive for a role in invasion.* These clones will be further investigated for altered expression levels in different tumor cell lines and tissues, and subsequently for their involvement in tumorigenesis, invasion, and metastasis. In the mean time, we will continue reconfirming the altered expression levels of the remaining potential Rac target genes by Northern blot analysis and subsequently further characterize them in the context of breast tumor progression.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of inducible cell lines expressing dominant active and dominant negative mutants of Rac.
- Successful completion of cDNA-RDA experiments to identify genes, which are up- or downregulated as a result of RacV12 or RacN17 expression.
- Implementation of the microarray technique to further analyze the difference products resulting from cDNA-RDA experiments.
- 300 clones out of a total of ca. 3,500 clones, derived from four independent cDNA-RDA experiments, were found to be differentially expressed by microarray analysis. These clones were sequenced, resulting in 85 independent gene fragments, of which 23 were novel. We found a total of 37 apparently upregulated and 48 downregulated candidate genes.

- *Eight clones have already been subjected to Northern blot analysis and reconfirmed for altered expression. These clones include CDO and VH16, which were found to be downregulated by RacV12. Furthermore, Myosin II, calcyclin, hBMP-4, and two novel clones of which ESTs are available, were found as clones upregulated by RacV12. Finally, a clone with homology to extracellular proteinase inhibitor HE4 was found to be upregulated by RacN17.*

REPORTABLE OUTCOMES

Manuscripts:

Schmitz, A., Lucito, R., and Van Aelst, L. (2000). Identification of Rac-regulated genes using cDNA-RDA in combination with microarraying. Methods in Molecular Biology (in press).

Schmitz, A., Lucito, R., and Van Aelst, L. (2000). Identification of novel Rac target genes in epithelial cells (manuscript in preparation).

Schmitz, A., Boettner, B., Govek, E,E., and Van Aelst, L. (2000). Rho-GTPases: Signaling, migration, and invasion. Exp. Cell. Res. Special issue on Cell Adhesion (review, in preparation).

Abstracts:

Schmitz, A., Lucito, R., and Van Aelst, L. Identification of Rac-regulated genes using cDNA-RDA. Poster at the '51. Mosbacher Kolloquium: GTP-binding Proteins: Central Regulators in Cell Biology', Mosbach, Germany, April 2 - 5, 2000.

Presentations:

Invited speaker (Linda Van Aelst) Gordon Research Conference, Singapore (Sept 5-10, 1999). Title: Function and signal transduction of Ras and Rac targets.

Invited speaker (Linda Van Aelst) Seminar, Van Andel Institute, Grand Rapids, MI (Nov. 30, 1999). Host: George Vande Woude. Title: Ras and Rac targets.

Invited speaker (Linda Van Aelst) Seminar, SUNY, Stony Brook (April 26, 2000). Host: Wadie Bahou. Title: Isolation of genes involved in Rac induced invasion and metastasis of breast carcinoma cells.

Invited speaker (Arndt Schmitz) Seminar, Biozentrum of the University of Basel, Switzerland (August 15, 1999). Host: Gerhard Schwarz. Title: Identification of genes regulated by the Small GTPase Rac using cDNA-RDA.

CONCLUSIONS

The involvement of Rac, a member of the Rho subfamily of small GTPases, in cellular processes such as proliferation, adhesion, and invasion is well established. To identify target genes of Rac,

we applied cDNA-RDA and microarray analyses. In particular, cDNAs prepared from cells induced for RacV12 and RacN17 expression were used as tester and driver against cDNAs from uninduced cells, to identify genes which are up- or downregulated as a result of Rac mutant expression. These experiments resulted in the identification of 85 independent gene fragments (among them 23 novel genes), of which 37 were apparently upregulated and 48 downregulated. The obtained genes could be categorized into the following different groups: genes encoding nuclear, ribosomal, mitochondrial, membrane associated, secreted and cytoskeletal proteins. To assure the differential expression of the above genes, we initiated Northern blot analysis. *Presently, we reconfirmed the difference in mRNA abundance of eight genes. These include: CDO and VH16, as clones downregulated by RacV12; myosin II, calcyclin, hBMP-4 and two novel genes of which ESTs are available, as clones upregulated by RacV12, and a clone with homology to extracellular proteinase inhibitor HE4 as a clone upregulated by RacN17. The outcome of these experiments is very promising and exciting, in particular, given that some of the clones identified have previously been associated with tumorigenesis and that the nature of some of the molecules, e.g. HE-4, is suggestive for a role in invasion.* To further establish the importance of these genes in breast tumor progression, they will be investigated for altered expression levels in different tumor cell lines and tissues and subsequently for their involvement in invasion and metastasis, by performing invasion and metastasis assays. In the mean time, we will continue reconfirming the altered expression levels of the remaining potential Rac target genes by Northern blot analysis. Based on our current data, we believe that amongst the genes identified, several of them will be involved in conferring invasive and metastatic potential at different stages during the tumor progression process. These genes may provide additional diagnostic markers, as well as provide new targets for therapy.

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APPENDICES

Figure 1. Principle of cDNA-RDA.
Figure 2. A representative example of cDNA-RDA.
Figure 3. Analysis of cDNA-RDA products by the microarray technique.
Figure 4. An overview of upstream activators and downstream signaling pathways of Rac.

Table 1. Different sample formats for microarraying.

Manuscript: Schmitz, A., Lucito, R., and Van Aelst, L. (2000). Identification of Rac-regulated genes using cDNA-RDA in combination with microarraying. Methods in Molecular Biology (in press).

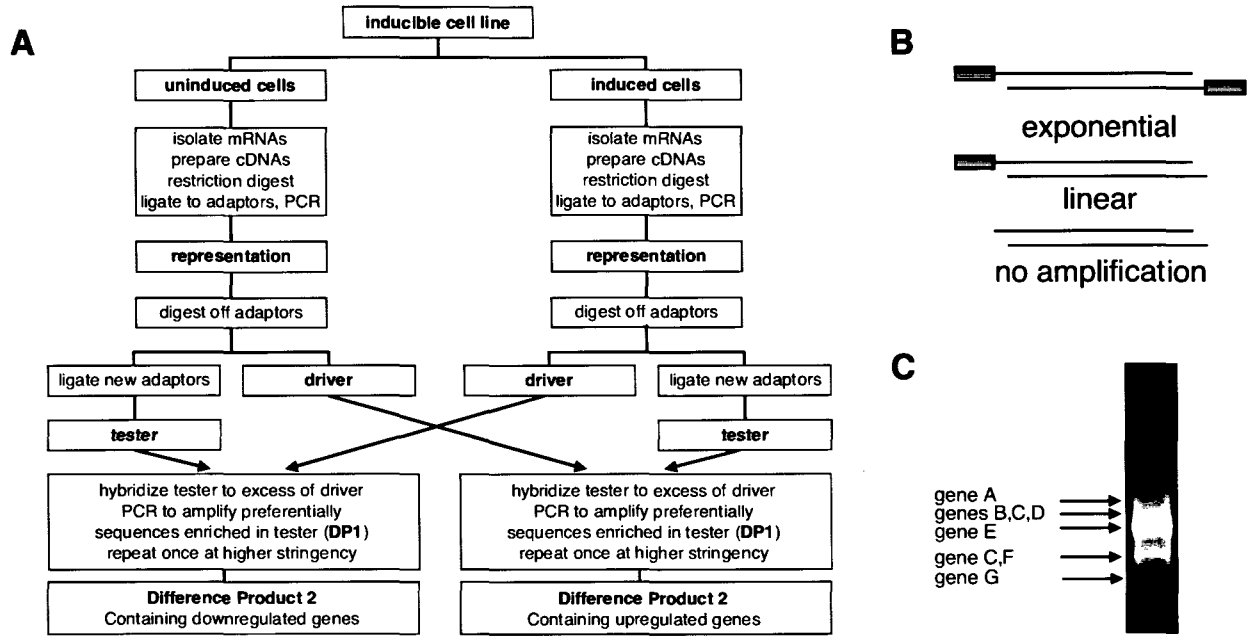

Fig. 1. Principle of cDNA-RDA. A) Representations are prepared from cells induced and uninduced for the gene of interest, by isolation of mRNAs, synthesis of cDNAs, restriction digest with DpnII, ligation to adaptors, and amplification by PCR. The adaptors are subsequently removed by digestion in order to use the representation as the driver in a cDNA-RDA experiment. To use the representation as the tester, the adaptors are cut off and replaced with newly ligated adaptors having a different sequence. Next, the tester from one sample is hybridized to an excess of driver from the other sample and sequences enriched in the tester are selectively amplified by PCR to obtain the difference product one (DP1). Finally, the DP1 is used as the tester in a new round of hybridization and amplification resulting in difference product two (DP2). Note that by performing two sets of reactions in parallel, using cells from each of the two samples once as tester and once as driver against the other sample, up- as well as down-regulated genes can be identified. **B)** The three possible outcomes of the tester/driver hybridization. If a sequence is unique to the tester, or present at a higher molar ratio in the tester than in the driver, it will be exponentially amplified. If a sequence is found in both driver and tester to equal amounts, only the strand from the tester population has the adaptor and the sequence will be linearly amplified. If the sequence is found only in the driver, neither strand contains the adaptor sequence and the sequence will not be amplified. **C)** A typical difference product after two rounds of cDNA-RDA visualized by agarose gel electrophoresis. A difference product consists of a series of visible bands superimposed on a 'smear'.

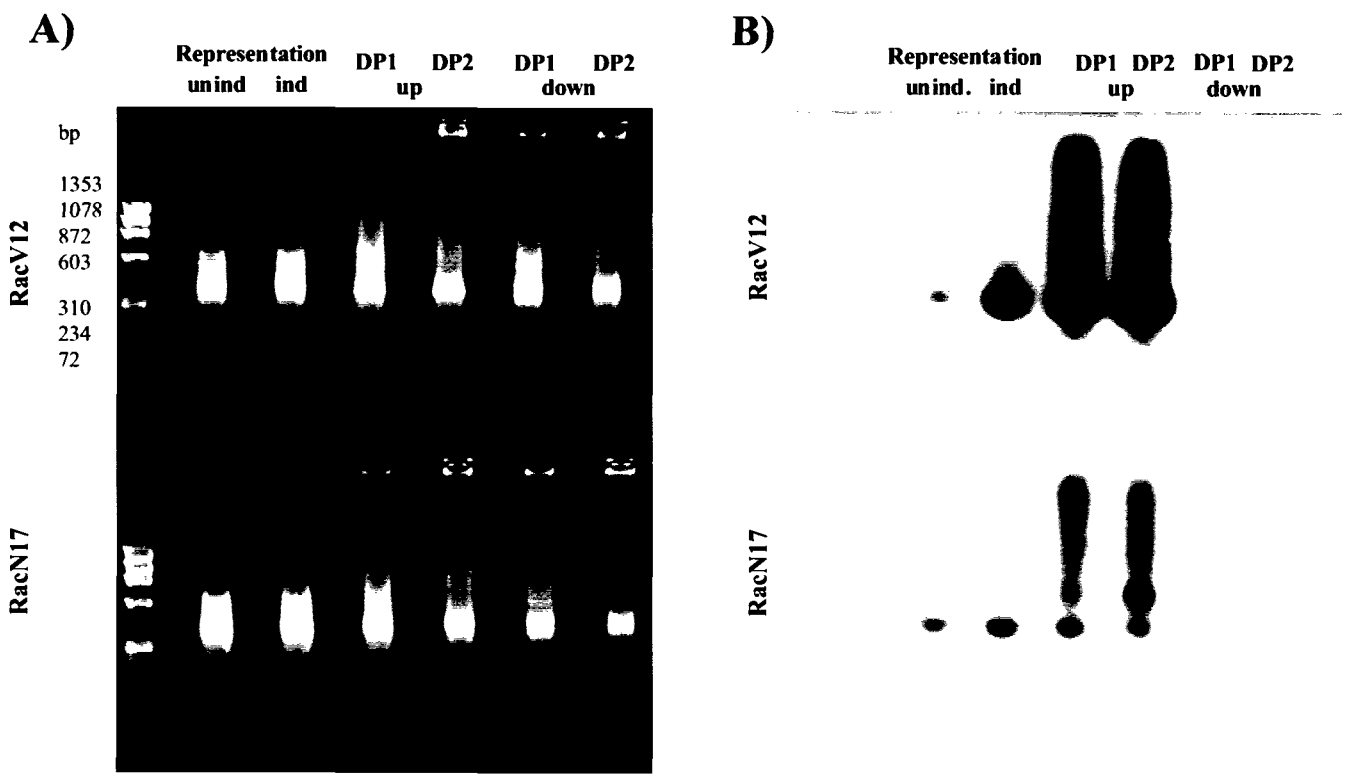

Fig. 2. A representative example of cDNA-RDA. **A)** Agarose gel electrophoresis of the original representations used for RDA, and the difference products obtained after the first (DP1) and second (DP2) cycles of RDA. Up (upregulated) denote the difference products obtained when using representations prepared from RacV12 or RacN17 induced cells as tester and uninduced cells as driver. Down (downregulated) denotes the difference products obtained when using representations prepared from uninduced cells and RacV12 or RacN17 induced cells as driver. **B)** Southern blot analysis of representations and difference products. The gel shown in A was transferred to a nylon membrane and probed with radioactive labeled Rac fragment.

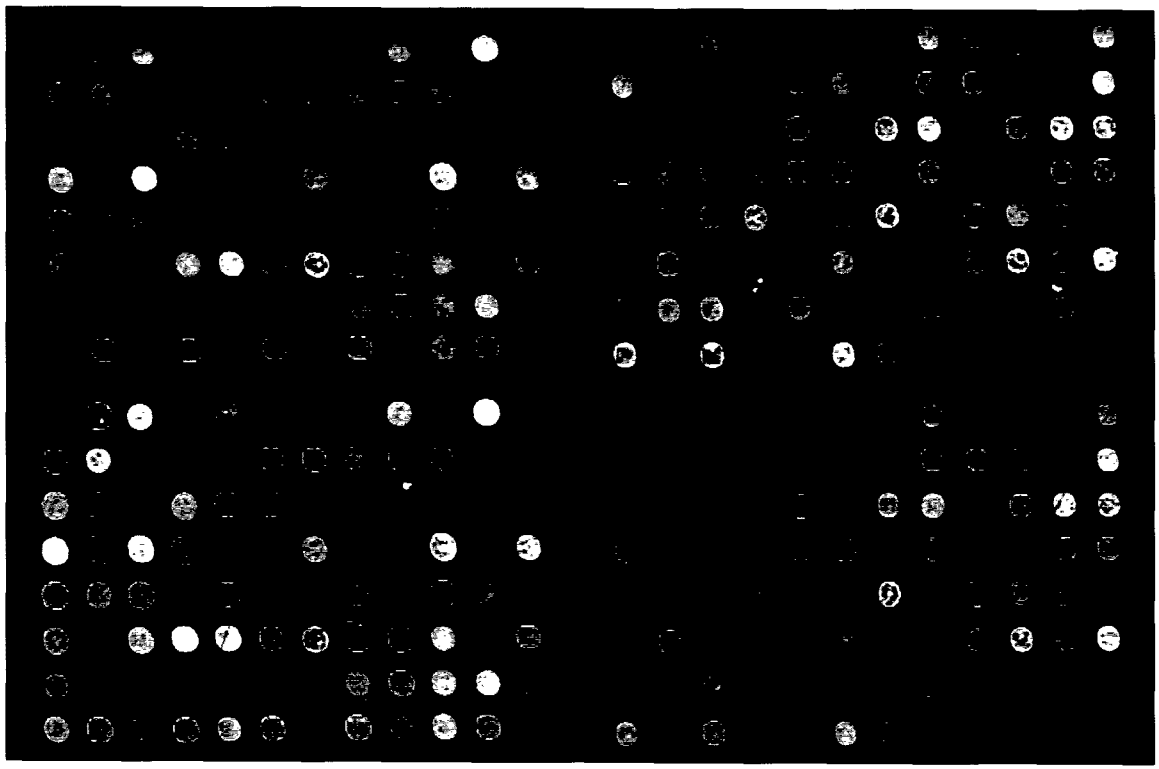

Fig 3. Analysis of cDNA-RDA products by the microarray technique. 190 clones derived from a cDNA-RDA experiment using cells induced for RacV12 expression as tester and uninduced cells as the driver were arrayed in duplicate. The array was then hybridized simultaneously to a representation prepared from RacV12 induced cells (labeled in green) and to a representation prepared from uninduced cells (labeled in red). The spots with different shades of green represent genes whose expression levels are upregulated as a result of RacV12 expression, whereas yellow spots are transcripts unaffected by RacV12 expression. Genes downregulated by RacV12 would be identified as red spots, but are absent as expected from the design of this particular experiment. Microarraying allows rapid screening of difference products as such to identify clones with the highest differential expression ratio.

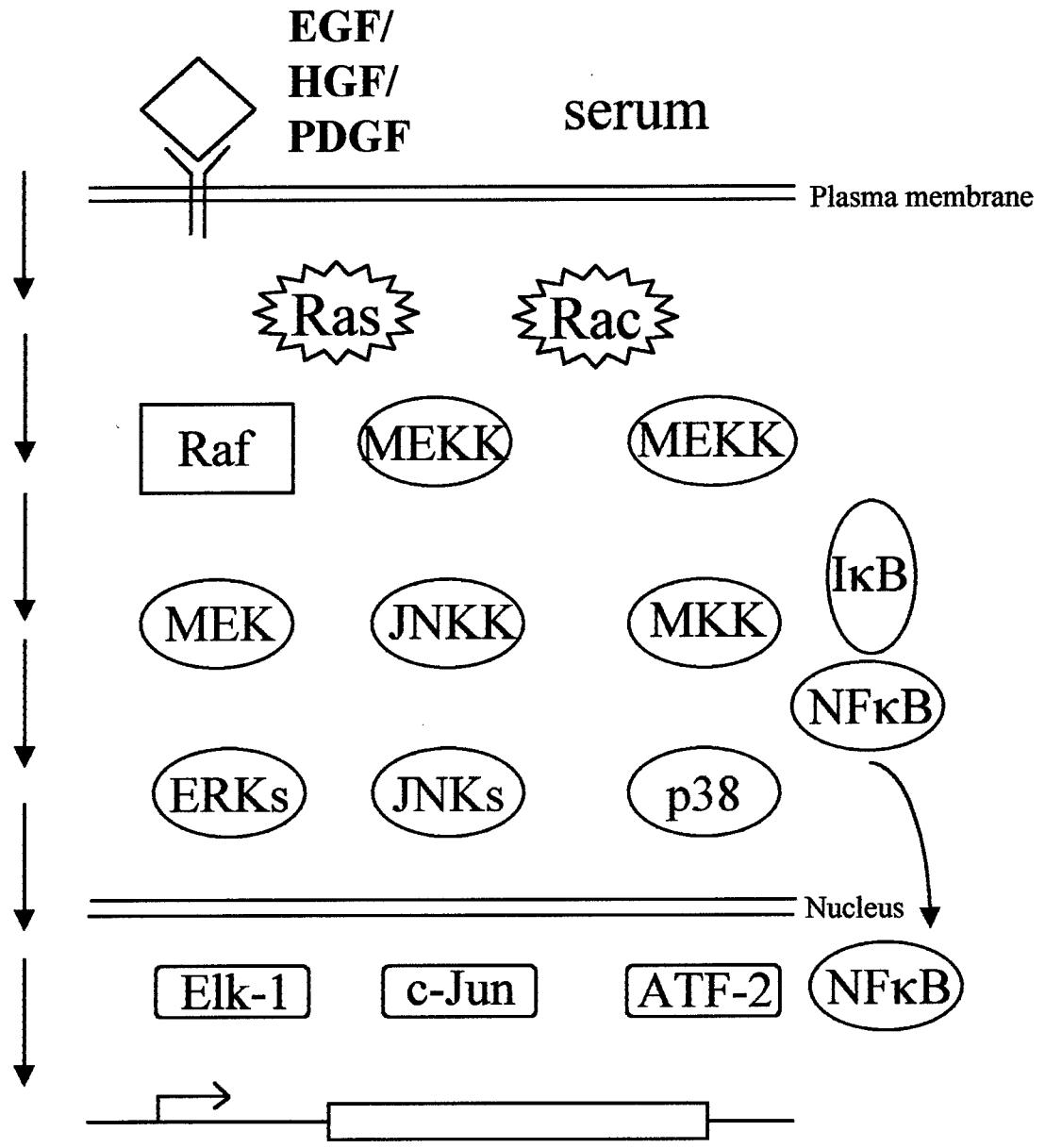

Fig. 4. An overview of upstream activators and downstream signaling pathways of Rac. Growth factors, such as EGF, HGF and PDGF are known to stimulate Rac activity, and subsequently Rac triggers the activation of ERK, JNK, p38 MAPK and NFκB. Highlighted in green and red are possibilities to stimulate and interfere with Rac signaling pathways respectively.

Table 1: Different sample formats for microarraying. Features corresponding to genes A to G were printed on glass slides and hybridized to samples obtained from cells induced and uninduced for RacV12 expression. Abbreviations are denoted as follows: RNA= total RNA probe; mRNA= poly(A)RNA probe; Repr= cDNA-representations; n= number of features/gene.

Gene	RNA	mRNA	Repr	n
A	3.0	4.9	6.7	9
B	0.5	0.6	0.4	6
C	1.4	2.0	2.7	4
D	1.3	1.1	1.2	2
E	0.7	1.0	0.8	3
F	4.4	6.5	7.8	1
G	3.7	4.7	6.2	2

Identification of Rac-regulated genes using cDNA - representational difference analysis (cDNA-RDA) in combination with microarraying

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1. INTRODUCTION

Small GTPases of the Ras superfamily are molecular switches which cycle between an active GTP-bound and an inactive GDP-bound state. They integrate signals from the cell surface to the nucleus, regulating important cellular activities. For example, Ras itself is activated when extracellular growth factors such as PDGF or EGF bind to their receptors at the cell surface. This activation of Ras leads ultimately to changes in the transcriptional activity of the cell, e.g. via the canonical MAPK cascade. Constitutively activated, mutant forms of Ras such as RasV12 are found frequently in human tumors and it is widely assumed that this oncogene acts via transcriptional activation of growth and proliferation pathways.

While the Rho family members, including Rho, Rac, and Cdc42, are best known for control of the actin cytoskeleton, they have also been linked to transcriptional activation. For example, activation of Rac triggers the activation of p38 and JNK MAPKs, as well as NFκB pathways [1]. Furthermore, numerous studies support a role for Rac in proliferation, invasion, and the control of cell adhesion. All these events occur over a longer time scale compared to the short-term changes of the actin cytoskeleton, and it is well assumed that these events require transcriptional activation. However, while pathways leading from Rac to the nucleus have been identified, information on genes regulated by Rac (or other members of the Rho GTPase family) remains scarce.

Within the last decade, several methods have been developed to identify changes in gene expression. These include serial analysis of gene expression (SAGE) [2], differential display (DD) [3, 4], representational difference analysis of cDNAs (cDNA-RDA) [5, 6], and suppression subtractive hybridization (SSH) [7, 8]. Noteworthy, each of the above techniques has its limitations. For example, Harris et al. identified differentially expressed genes in Aflatoxin B1 - treated hepatocytes, using in parallel DD, cDNA-RDA, and SSH, and each of these three methods found a small, non-overlapping set of differentially expressed genes [9]. Even more recently, expression analysis utilizing microarray technology has become available [10]. This technique

depends on the availability of reliable cDNA clones that can be arrayed, whereas the former methods allow the identification of novel sequences in incompletely characterized organisms. One major advantage of cDNA-RDA compared to other methods is its low level of false positives, because RDA eliminates those fragments which are present in both DNA populations. Furthermore, cDNA-RDA does not require sophisticated equipment.

We recently used cDNA-RDA to identify genes differentially expressed between cell lines induced and not induced for expression of the small GTPase Rac under the control of an inducible promoter. cDNA-RDA is a modified form of RDA, a PCR-based differential cloning method (Fig. 1) [11, 12]. In this technique, one DNA population (called the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester. RDA relies on the use of representations of the DNAs of interest. In brief, a representation is prepared by restriction digestion of the DNA (e.g. *Dpn*II in the case of cDNA-RDA), ligation of adaptor oligonucleotides, and subsequent PCR amplification.

One major task is the analysis of the difference product resulting from the cDNA-RDA experiment (Fig. 1c). To identify the most promising candidate genes, we and others [13] used the microarray technique as detailed below (Fig. 2). Only the clones with the highest differential expression as judged by microarraying were sequenced and further pursued. By doing so, we were able to increase significantly the number of clones screened per cDNA-RDA experiment.

2. MATERIALS

2.1. Equipment

1. A PCR machine capable of handling 0.5 ml PCR tubes to do cDNA-RDA (e.g. Perkin Elmer DNA Thermal Cycler 480).

2. A PCR machine which can hold 96-well plates (e.g. Perkin Elmer GeneAmp 9600) or up to four 96-well plates simultaneously (e.g. MJ Research PTC-225) to process the samples for microarraying.
3. A speedvac (e.g. Savant DNA 100).
4. 8-channel pipetters such as Labsystems Finnpipette 4510000 (0.5 - 10 µl) and 4510020 (5 - 50 µl) and a repeat pipette (e.g. Brinkmann Eppendorf 22260006) are very helpful for working with 96-well plates.
5. 96-well PCR plates are obtained from Perkin Elmer (N801-0560), 96-well plates for dilutions of DNA samples are retrieved from Nunc, while 96-well plates for microarraying (with V-shaped bottom) are obtained from Corning-Costar. Plates are covered either with caps (during PCR, Perkin Elmer N801-0535) or sealing film (Sigma Z36,968-3).
6. UV crosslinker (Stratalinker 2400, Stratagene).
7. Minifold I dot – blotting apparatus (Schleicher and Schuell SRC096/0).
8. Hybond-N+ nylon membrane (Amersham Pharmacia Biotech RPN203B).
9. Microcon YM-30 ultrafiltration columns (Amicon) to purify and concentrate labeling reactions.
10. A vacuum oven.
11. A microarrayer, e.g. Cartesian PixSys 5500 (Cartesian Technologies, Irvine, CA).
12. Pins for the arrayer (Chipmaker 2, Telechem International).
13. Silanated glass slides (Corning).
14. A humidified hybridization chamber (Telechem International).
15. A scanner suitable for microarray fluorescence detection, such as GSI Lumonics ScanArray3000 or Axon GenePix4000.
16. Analysis software: We made use of ScanAlyze (Stanford University) or Axon GenePix to determine features and for the quantitative analysis of the resulting TIFF files.

2.2. Enzymes and reagents

1. Organic solvents such as 70 % and 100 % ethanol (EtOH), isopropanol (iPrOH), chloroform, dimethylsulfoxide (DMSO), and phenol / chloroform / isoamylalcohol (25 / 24 / 1) saturated with TE pH 8.0 (Sigma P 2069).
2. FastTrack mRNA isolation kit (Invitrogen K1593-02).
3. CopyKit cDNA synthesis kit (Invitrogen L1311-03).
4. *Dpn*II restriction enzyme and 10 x *Dpn*II buffer (New England Biolabs 543L).
5. 10 mg / ml tRNA (Sigma R 8759) is used as a carrier during precipitations of small amounts of DNA.
6. Sheared salmon sperm DNA (Stratagene 201 190, diluted to 50 ng / µl).
7. DNA molecular weight marker ϕX174 *Hae*III digest (New England Biolabs 302-6L).
8. T4 ligase and 10 x T4 buffer (New England Biolabs 202S).
9. The primers listed in Table 1.
10. AmpliTaq polymerase, 25 mM $MgCl_2$, and 10 x Taq buffer without $MgCl_2$ (5 U / µl, Perkin Elmer N8080-153).
11. dNTPs (100 mM, Roche Molecular Biochemicals 1 696 064).
12. Mung Bean Nuclease (MBN) and 10 x MBN buffer (New England Biolabs 250S).
13. Qiaquick gel extraction kit (Qiagen 28704).
14. *Bam*HI restriction enzyme, 10 x *Bam*HI buffer, and 10 mg / ml BSA (New England Biolabs 136S).
15. Calf intestinal phosphatase (CIP) and 10 x CIP buffer (New England Biolabs 290S).
16. Qiaquick PCR purification kit (Qiagen 28104).
17. DNA polymerase I Klenow fragment and 10 x reaction buffer (Amersham Pharmacia Biotech E2141).

18. Cy3-dCTP and Cy5-dCTP for probe labeling (Amersham Pharmacia Biotech PA53021 and PA55021).
19. dRhodamine dye terminator kit for non-radioactive DNA sequencing (Perkin Elmer 403045).

2.3. Buffers

1. 3 M sodium acetate (NaAc), pH 5.2.
2. Elution buffer (EB; 10 mM Tris/HCl pH 8.5).
3. 6 x GLB (Gel Loading Buffer, 30 % glycerol and 0.25 % bromophenol blue. Not necessary to autoclave.)
4. 500 mM Tris/HCl pH 8.9 (It is important to use Tris base adjusted to pH with HCl instead of Tris Hydrochloride adjusted to pH with NaOH.)
5. 100 mM $MgCl_2$.
6. 200 mM $(NH_4)_2SO_4$, sterile filtered.
7. TE Buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).
8. 10 M ammonium acetate (NH_4Ac).
9. EE Buffer (30 mM EPPS (Sigma E 1894) pH 8.0, 3 mM EDTA pH 8.0), sterile filtered.
10. 5 M NaCl.

2.4. PCR Buffers

PCR buffers are prepared from autoclaved or sterile filtered stock solutions and autoclaved water (Note 1). All PCR buffers are made immediately before use and stored on ice.

1. PCR buffer 1 is used in Subheading 3.2.1.3. for the preparation of representations. This buffer consists of 67 mM Tris pH 8.9, 4 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mg/ml BSA, 16 mM $(NH_4)_2SO_4$, 320 µM each dNTP, 1.25 µM primer RBgl24, and 0.04 U / µl (15 U / tube) Taq.

2. PCR buffer 2 is identical to buffer 1, except that the primer and the Taq polymerase are added later. This buffer is used for the first PCR in each round of cDNA-RDA described in Subheadings 3.2.2.3. and 3.2.3., step 4.
3. PCR buffer 3 is also identical to buffer 1, except that primer NBgl24 (in Subheading 3.2.2.4.) or IBlg24 (in Subheading 3.2.3., step 4) are used. This buffer is employed during the second PCR in each round of cDNA-RDA.
4. PCR buffer 4 is used for the single colony PCR described in Subheading 3.3.3. This buffer consists of 12 % DMSO in 1 x Taq buffer supplemented with 2 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM primer SP6R, 0.5 µM primer T7E, and 0.025 U / µl (0.625 U / well) Taq polymerase.
5. PCR buffer 5 is a standard PCR buffer used in Subheading 3.3.5. for reamplification. Buffer 5 is identical to buffer 4, except that the DMSO is omitted.

3. METHODS

A detailed description of the different steps in cDNA-RDA and the analysis of the difference product by microarraying is given. More common techniques used throughout our study, such as Western, Northern, and Southern blot analysis, are not described here in detail.

3.1. Preparation of cDNAs

In order to obtain meaningful results by cDNA-RDA, the samples used for the preparation of tester and driver need to be selected carefully. It is best to differ in only one parameter and to compare two samples matched as close as possible. For this reason, we made use of an inducible system for the expression of the target gene [14-16].

1. Perform time courses and titrate the concentration of inducer to establish optimal induction conditions for the selected inducible clones.
2. Grow approximately 5 x 10 ^ 7 cells of one selected clone in one incubator. Split cells into two halves and induce one half of the population. For example, in the case of MDCK cells with

expression of RacV12 under the control of the tetracycline-off system, two days in the absence of doxocycline were sufficient to induce expression of RacV12 to the level of endogenous Rac [17]. Continue to use the same batch of medium for tester and driver populations. Harvest approximately 1 x 10 ^ 8 each of uninduced and induced cells by scraping into ice-cold PBS. Process immediately or store the cell pellet at -80 °C. Before continuing, check by Northern or Western blot analysis to see whether induction was successful.

3. Isolate mRNA from cells induced and not induced for expression of the gene of interest. Several commercial kits are available. We used the Fast Track kit for mRNA isolation and the CopyKit for cDNA preparation (both from Invitrogen). A yield of approximately 3 µg mRNA / 10 ^ 7 cells can be expected, depending on the cell type. Use 5 µg mRNA to generate approximately 7 µg cDNA (note 2).

3.2. cDNA-RDA

3.2.1. Preparation of Representations

Representations are obtained by digestion of the cDNA sample of interest with a frequently cutting restriction enzyme such as *Dpn*II (GATC), followed by ligation to short adaptor oligonucleotides and amplification by PCR, using the same sequences as primers. Representations to be compared to each other by cDNA-RDA have to be prepared simultaneously.

3.2.1.1. Digestions of the cDNAs

1. Digest the two cDNA samples derived from the induced and the uninduced cells in parallel. Each digest consists of 1.2 µg of cDNA in 10 µl 10 x *Dpn*II buffer, 10 µl 10 U / µl *Dpn*II, and H_2O to a total volume of 100 µl overnight (16 h) at 37 °C (note 3).
2. Add 1 µl 10 µg / µl tRNA, vortex, and extract twice with 100 µl phenol/chloroform. Add 10 µl 3M NaAc pH 5.2 and 330 µl 100 % EtOH (kept at - 20 °C), vortex, and incubate at - 70 °C for 10 min.

3. Spin 10 min at 14 k rpm at 4 °C, and wash the pellets with 500 µl 70 % EtOH (kept at -20 °C). Spin again, remove wash solutions as completely as possible by pipetting. Dry in a speedvac at medium heat and resuspend carefully in 12 µl EB (note 4).
4. Transfer 2 µl to a tube containing 8 µl H_2O and 2 µl 6 x GLB. Load the samples onto a 2 % agarose gel, side-to-side with 200 ng undigested cDNA (to check digest), a dilution series of 50 - 100 - 200 ng sheared salmon sperm DNA (to check recovery and concentration), and 200 ng of ϕX174 *Hae*III as the marker.

3.2.1.2. Ligation

The ligation is preceded by an incubation step ensuring optimal conditions for the adaptors to hybridize to the cDNA fragments as follows:

1. To the remaining 10 µl of each cDNA digest, add 7.5 µl 62 µM RBgl24, 7.5 µl 62 µM RBgl12, 3 µl of 10 x T4 buffer, and 2 µl H_2O.
2. Mix well and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55 °C.
3. Unplug the heatblock, transfer the block with the two tubes to the cold room, and allow the temperature to decrease to 10 - 15 °C over the next 90 min (check).
4. Add 1 µl T4 ligase to each tube, mix by pipetting, and incubate for 16 h at 12 °C.

3.2.1.3. Amplification by PCR

To ensure equal conditions during preparation of representations, these PCRs must be performed in parallel in the same PCR machine using the same mastermix for both sets of reactions.

1. Add 970 µl of TE supplemented with 200 µg/ml (20 µl of 10 µg / µl) tRNA to each of the two ligations, vortex, and store on ice.
2. Label two times ten 0.5 ml PCR tubes, a set each for the induced and the uninduced sample, respectively. Prepare 8 ml of PCR buffer 1 and aliquot 360 µl into each of the 20 PCR tubes.

Add 40 µl from each of the two diluted ligations to the ten corresponding tubes. Vortex each tube briefly before adding one drop mineral oil to each tube.

3. Cycle all 20 tubes together 20 times in a two-temperature PCR cycle (1 min at 95 °C and 3 min at 72 °C). Finish the reaction by a final extension at 72 °C for 10 min.
4. To check the outcome of the PCR, run 8 µl of the reaction from each tube + 1.6 µl 6 x GLB on a 2 % agarose gel, using ϕX174 *Hae*III as the marker and 200 - 300 - 400 ng sheared salmon sperm DNA to check yield (approximately 300 ng per lane can be expected). All ten aliquots prepared from the induced sample should look identical. Also, all ten aliquots from the uninduced sample should look identical among themselves. Often, a band pattern specific for each of the two samples is observed.
5. Withdraw the PCR reactions under the mineral oil and collect each of the two samples (in total 4 ml from the ten matching tubes) in a 15 ml Falcon tube. Extract both pooled samples twice with 3 ml phenol/chloroform. Add 400 µl 3M NaAc and 8 ml iPrOH. Incubate for 15 min at 4 °C (or overnight at - 20 °C), and spin 30 min at 4 °C.
6. Wash pellets once with 5 ml 70 % EtOH (kept at - 20 °C), recentrifuge for 5 min, remove supernatants, and dry pellets in a desiccator. Redissolve pellets in 500 µl TE. Check recovery by diluting 2 µl sample 1:10 with TE and running 2, 5, and 10 µl on a 2 % agarose gel with 100 - 250 - 500 ng salmon DNA as standards.
7. Estimate the amount of DNA on the gel and use it to calculate the total amount of representation recovered. Approximately 150 µg for each of the two representations prepared can be expected. This corresponds to ca. a 400-fold amplification, since the representations were made from 0.4 µg template each (note 5).

3.2.1.4. Removal of the adaptors by digestion

Before the representations can be used in a cDNA-RDA reaction, the adaptors at their ends must be cleaved by restriction enzyme digestion.

1. Use 100 µg of each of the two representations prepared in Subheading 3.2.1.3. for a digest to remove adaptors. In a 2 ml eppendorf tube, add 100 µg representation to a reaction mix consisting of 100 µl 10 x *Dpn*II buffer, 100 µl 10 U / µl *Dpn*II, and water to 1 ml final volume. Incubate overnight at 37 °C, preferentially in an incubator.
2. Extract twice with 1 ml phenol/chloroform. Add 100 µl 3M NaAc and 1 ml iPrOH. Incubate for 15 min at 4 °C (or overnight at - 20 °C), and spin the two tubes at 14 K rpm at 4 °C for 15 min. Wash pellets once with 1 ml 70 % EtOH (kept at - 20 °C), and carefully dry pellets in a speedvac. Redissolve carefully in 100 µl TE by pipetting and vortexing.
3. Check recovery and completeness of digestion by preparing 10 µl of a 1:10 dilution and run 1, 2.5, and 5 µl samples on a 2 % agarose gel. Include 200 ng samples of the undigested representations (Subheading 3.2.1.3.). The band pattern before and after digestion should be identical, except that after digestion it will be shifted slightly downward due to the removal of 48 bp of adaptor sequence. Use 100 - 250 - 500 ng salmon DNA to check yield. Adjust concentrations to 500 ng / µl with TE, pipet, and revortex.

3.2.2. First round of cDNA-RDA

Since cDNA-RDA identifies only samples upregulated in one sample relative to the other, we perform two cDNA-RDA experiments in parallel (see Fig. 1). To identify downregulated genes, add the representation derived from induced cells as the driver to the tester derived from uninduced cells. To identify upregulated genes, use the representation derived from uninduced cells as the driver, and add it to the tester derived from induced cells. In this experiment, it is advisable to add the induced gene of interest back to the driver, since this sequence may otherwise constitute a major part of the difference product (see note 6).

3.2.2.1. Ligation of new adaptors to tester

1. Set up in parallel two ligations to obtain testers from each of the two samples. Each ligation contains 2 µl of 500 ng / µl digested representation (see Subheading 3.2.1.4.), 7.5 µl 62 µM NBgl24, 7.5 µl 62 µM NBgl12, 3 µl 10 x T4 buffer, and 10 µl H_2O.
2. Mix well, and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55 °C. Unplug the heatblock and transfer the block with the tubes to the cold room and allow the temperature to fall to 10 - 15 °C during the next 90 min.
3. Add 1 µl T4 Ligase, mix by pipetting, and incubate for 16 h at 12 °C.
4. Add 70 µl TE supplemented with 20 µg / ml tRNA, and store on ice.

3.2.2.2. Hybridization

1. Adjust one thermoblock with glycerol or mineral oil filled holes to 67 °C and another one to 98 °C.
2. For each of the two reactions, add 80 µl of 500 ng / µl (40 µg) digested representation, corresponding to the driver (see Subheading 3.2.1.4.), to 40 µl of 10 ng / µl (0.4 µg) representation ligated to new adaptors (see Subheading 3.2.2.1.; the tester). Vortex the eppendorf tubes and extract once with 120 µl phenol / chloroform. Add 30 µl 10 M NH_4Ac, vortex, and add 380 µl EtOH (kept at - 20 °C). Incubate at - 70 °C for 10 min, warm the samples for 2 min at 37 °C, and spin at 4 °C at 14 K rpm for 20 min. Wash twice with 500 µl 70 % EtOH, and dry carefully in a speedvac (up to 2 min, without heating).
3. Resuspend each pellet in 5 µl EE by pipetting and vortexing four times for 30 sec each. Spin down very briefly and carefully add 35 µl mineral oil. Denature DNA at 98 °C for 4 min, add 1.5 µl 5 M NaCl to the aqueous phase by pricking through the mineral oil with the pipett tip, and incubate at 67 °C for 20 h.

3.2.2.3. First PCR

1. Remove mineral oil from the hybridizations (see Subheading 3.2.2.2.). Add tRNA (8 µl 5 µg / µl) to the hybridization mix. Mix by pipetting, add 390 µl TE, and vortex.

2. Prepare 1.5 ml of PCR buffer 2, distribute 352 µl each into four PCR tubes. Add 40 µl (4 µg total DNA) from the one hybridization to two of the four tubes and 40 µl from the other hybridization to the remaining two tubes, and place them in a PCR machine kept at 72 °C.
3. Add 3 µl 5 U / µl Taq polymerase to each of the four tubes, vortex, and incubate for 5 min at 72 °C, during which time the 3' ends of the adaptor sequences will be filled in. Add 10 µl of 62 µM primer NBgl24 to each tube, vortex, add mineral oil, and cycle 10 times in a two-temperature PCR (keep at 95 °C for 1 min and at 72 °C for 3 min).

3.2.2.4. MBN digest and second PCR

The PCR step described in Subheading 3.2.2.3. selectively amplifies the sequences enriched in the tester. To improve amplification of tester-specific sequences during the second PCR amplification, the single stranded driver DNA is digested by MBN treatment.

1. Place a waterbath at 30 °C and a heat block at 98 °C.
2. Pool the identical samples obtained after the first PCR (Subheading 3.2.2.3.). Extract once with 600 µl phenol/chloroform and once with 600 µl chloroform. In a 2 ml eppendorf tube, add 80 µl 3M NaAc and 1 ml iPrOH.
3. Incubate for 1 h at - 20 °C, spin 15 min at 14 K rpm at 4 °C. Wash pellets once with 500 µl 70 % EtOH (kept at - 20 °C), recentrifuge for 5 min, and remove supernatants.
4. Dry pellets in a speedvac and redissolve carefully in 40 µl EB by pipetting and vortexing. Prepare 50 µl 2 x MBN buffer.
5. Incubate 20 µl from each of the two samples in parallel with 20 µl 2 x MBN buffer and 2 µl 10 U / µl MBN enzyme for 30 min at 30 °C.
6. Neutralize with 160 µl 50 mM Tris pH 8.9 and inactivate the MBN for 5 min at 98 °C, then store on ice.

7. Prepare 1.5 ml of PCR buffer 3, distribute 360 µl into four PCR tubes. Add 40 µl from each sample from the preceding step to two PCR tubes. Do not combine the samples at this step. Vortex, add mineral oil, and perform 20 PCR cycles of 1 min at 95 °C and 3 min at 72 °C.
8. To evaluate the outcome of the PCR, run 10 µl of the reaction from each tube + 2 µl 6 x GLB on a 2 % agarose gel and use 100 - 200 - 300 ng sheared salmon sperm DNA to check yield (a yield of approximately 200 ng / 10 µl can be expected). In the case of a low yield, supplement each tube with 3 µl of fresh Taq polymerase and perform three additional PCR cycles.
9. Pool the identical samples and extract them once with 600 µl phenol/chloroform and once with 600 µl chloroform. In a 2 ml eppendorf tube, add 80 µl 3M NaAc and 1 ml iPrOH. Incubate overnight at - 20 °C.
10. Centrifuge for 15 min, 14 K rpm at 4 °C. Wash pellet once with 500 µl 70 % EtOH (kept at - 20 °C), and dry the pellets in a speedvac.
11. Redissolve the pellets carefully in 100 µl TE by pipetting and vortexing. Prepare 20 µl of a 1:5 dilution and run 2.5, 5, and 10 µl on a 2 % agarose gel. Include lanes with 100 - 200 - 300 - 400 ng salmon DNA to check yield (approximately 20 µg / each sample is to be expected). Adjust the concentration to 100 ng / µl and store the difference product 1 (DP1) at - 20 °C.

3.2.3. Second round of cDNA-RDA

The second round of cDNA-RDA is basically a repetition of the first round, incorporating the following changes: The product of the first round of cDNA-RDA (DP1) is used as the tester, while the driver is still the digested representation. The driver to tester ratio is increased from 100:1 to 800:1. Also, a new set of primers is used.

1. Remove the NBlg24 adaptors from 5 µg of DP1 with *Dpn*II following the protocol provided in Subheading 3.2.1.4. Resuspend after precipitation in 50 µl TE. Check the yield by agarose gel electrophoresis and adjust the concentration to 20 ng / µl with TE.

2. Ligate new adaptors to 100 ng (5 µl) of digested DP1, using the same conditions and concentrations of reagents as described in 3.2.2.1., but using IBgl24 and IBgl12 instead of NBgl24 and NBgl12. Incubate overnight, add 50 µl TE supplemented with 20 µg / ml tRNA, and store on ice.
3. Mix 40 µl (50 ng) of tester just ligated to new IBgl adaptors and 80 µl of 500 ng / µl (40 µg) driver (prepared in Subheading 3.2.1.4.). Hybridize, following the instructions given in Subheading 3.2.2.2.
4. Perform first PCR, MBN digest, and second PCR as outlined in Subheading 3.2.2.3. and Subheading 3.2.2.4., substituting primer IBgl24 for NBgl24. Adjust the concentration to 100 ng / µl and store the difference product 2 (DP2) at - 20 °C (note 7).

3.3. Analysis of the obtained difference products

3.3.1. Southern Blot analysis

After completion of the cDNA-RDA experiment (which will take approximately 2 to 3 weeks), the difference products (DP2) are analyzed. A first standard control is performed by Southern Blot analysis, using the induced gene (e.g. Rac) as a positive control to probe equal amounts of each driver and tester representation, DP1, and DP2 transferred to a nylon membrane. If looking for genes downregulated by Rac, the hybridization signal from the Rac probe should be stronger in the driver than in the tester and absent from both DP1 and DP2. If looking for genes upregulated by Rac, the hybridization signal should be stronger in the tester than in the driver and more stronger in both DP1 and DP2 compared to tester. This signal is likely to be detected, although to a lesser extent, even when the induced gene was added back to the driver (see Note 6).

If the blot indicates that the cDNA-RDA experiment was successful, the individual clones contained in the DP2 are analyzed. The DP2 library is subcloned, individual inserts are amplified and revalidated by microarraying in order to sequence only the most promising candidate genes.

3.3.2. Subcloning

1. Digest 1 µg DP2 (see Subheading 3.2.3.) with *Dpn*II as described in Subheading 3.2.1.4.
2. After redissolving in 20 µl EB, add 4 µl 6 x GLB and run at low voltage on a 2 % agarose gel ca. 4 cm distance from the wells. With a fresh scalpel, cut out the whole lane from 800 – 100 bp, leaving the cleaved adaptors behind.
3. Use the Qiaquick gel extraction kit to recover the DNA and ligate the adaptor-free DP2 to a bacterial expression vector (e.g. pGEM7Zf(-)) digested with *Bam*HI and dephosphorylated with CIP) as described in Subheadings 3.2.1.2. and 3.2.2.1. Transform into highly competent *E. coli* and plate the library.

3.3.3. Single colony PCR

Individual clones are directly amplified from the bacterial colonies by using the bacteria as the template in a PCR reaction with primers directed against the sequences surrounding the multiple cloning site of the vector. We suggest to process four 96 well plates from each DP2 library.

1. Prepare 2.75 ml of PCR buffer 4 (note 8). Pipette 325 µl into each well of the first column of a 96-well PCR plate. With an 8-channel pipetter, distribute 25 µl into each of the wells across the plate.
2. Pick a single white colony from the plate using a sterile pipet tip, and dip the tip into a well of the PCR plate. Repeat 94 times, inoculating each well with an individual white colony picked at random using fresh tips. Include one well as a negative control without any template.
3. Amplify by PCR using a three temperature program (5 min at 94 °C for initial denaturation; 25 cycles of 1 min each at 94 °C, 60 °C, and 72 °C; additional 10 min at 72 °C for final elongation and hold at 4 °C). Run 5 µl sample, 5 µl H_2O, and 2 µl 6 x GLB on a 2 % agarose gel with ϕX174 *Hae*III as the standard, and photograph. Store remainder of the PCR reactions at - 20 °C.

3.3.4. Exclusion of cDNA clones which are identical to the induced gene of interest

It is to be expected that the induced gene (e.g. Rac) will be found as a difference product if the tester is derived from induced cells, even, as mentioned above, when the induced gene was added back to the driver (see also note 6). To prevent sequencing these clones, we identified them by dot-blotting experiments.

1. Prepare a 96-well plate with 10 µl TE in each well. Add 1 µl of each of the PCR reactions from Subheading 3.3.3. into the corresponding wells.
2. Following the manufacturer's instructions, place a Hybond-N+ nylon membrane in the minifold I dot-blotting apparatus with and apply vacuum. Load your DNA samples in the corresponding wells. Fix the DNA to the membrane, e.g. by UV-crosslinking.
3. Prepare 50 ng of radiolabeled double-stranded DNA probe from your induced gene (e.g. full length Rac1) and hybridize to the dot-blot filter.

3.3.5. Re-amplification for microarraying

The amplified clones need to be re-amplified in order to obtain sufficient amounts for microarraying as well as to ensure that the amplification was specific.

1. Prepare 10 ml of PCR buffer 5. Add 100 µl each to the wells of a 96-well PCR plate. With an 8-channel pipetter, add 1 µl each from the PCR reactions (see Subheading 3.3.3.) into the corresponding well. Amplify by PCR using the following program: 1 min at 94 °C for initial denaturation; 25 cycles of 1 min each at 94 °C, 60 °C, and 72 °C; additional 10 min at 72 °C for final elongation; hold at 4 °C).
2. Run 5 µl of each of the PCR products and 1 µl 6 x GLB on a 2 % agarose gel with ϕX174 *Hae*III as the standard, and photograph. Transfer the samples to a Corning 96-well plate, supplement with 10 µl 3 M NaAc and 200 µl EtOH, and keep for 16 h at -20 °C.
3. Centrifuge for 20 min at + 4 °C and 3750 rpm. Aspirate supernatants, wash with 100 µl 70 % EtOH, spin for 5 min, and aspirate supernatants. Dry the pellets in a vacuum oven at maximal 50 °C, resuspend in 15 µl of 3 x SSC per well, and store at -20 °C.

3.3.6. Microarraying

In our microarraying experiments, the clones derived from a cDNA-RDA experiment are arrayed on a chip. Representations from each driver and tester are labeled with a fluorophore, mixed, and then hybridized simultaneously to the arrayed candidate genes.

3.3.6.1. Array preparation

1. Array the re-amplified inserts from Subheading 3.3.5. on commercially prepared silanated slides, using a Cartesian PixSys 5500 or equivalent.
2. Place the array in a humidified chamber for 3-5 min to hydrate the spots. Crosslink the slide by UV irradiation with 60 mjoules in a UV crosslinker. Rehydrate the slide in the humidified chamber and snap dry by heating on the surface of a hot plate for several seconds. Wash the chip in 0.1 % SDS for approximately 10 sec, in deionized water for ca. 10 sec, and then denature the chip in boiling MilliQ water for ca. 1 - 2 min. After denaturation, immediately immerse the array in ice-cold benzene-free ethanol for several seconds. Take out and allow the chip to dry. Perform the wash procedure from the SDS to the ice-cold ethanol also with the cover slips to be used with the arrays.

3.3.6.2. Sample preparation

1. In parallel, denature 10 μg each of the representations derived from induced and uninduced cells in the presence of 5 μg of a random nonamer primer in a total of 100 μl H_2O. Use either representations ligated to adaptors (see Subheading 3.2.1.3.) or with the adaptors cleaved off (see Subheading 3.2.1.4.).
2. To each sample, add 12 μl of 10 x Klenow buffer and supplement with 33μM dNTPs, 10 nmoles of either Cy3 or Cy5, and 4 units of Klenow fragment. Incubate the reactions at 37 °C for 2 h.
3. Combine the two reactions and remove unincorporated nucleotides by centrifugation through a Microcon YM-30 ultracentrifugation column according to the manufacturer's instructions.

Adjust the eluate containing the labeled sample to a volume of 15 µl and a concentration of 3 x SSC and 0.2 % SDS, and denature by heating to 95 °C for 5 min.

4. Carefully place the 15 µl of labeled representations on the array prepared in Subheading 3.3.6.1. and slowly place a cover slip on the array. Insert into hybridization chamber according to manufacturer's instructions and incubate in the dark at 67 °C overnight.
5. After the overnight hybridization, disassemble the hybridization chamber and wash the array at room temperature in 0.1 % SDS, 0.2 x SSC for 90 sec, followed by a 30 sec wash in 0.2 x SSC and a final 30 sec wash in 0.05 x SSC.
6. After washing, arrays are imaged in a scanner. Images are saved as TIF files and feature definition and quantitative analysis are performed with either ScanAlyze or Axon GenePix. This analysis yields a text file that is imported into Microsoft Excel or Access for further analysis.

3.3.7. Non-radioactive sequencing of PCR products

Sequence all clones which are found to be differentially expressed based on microarraying (e.g., fluorescence ratio greater than 2). However, do not sequence the Rac clones already identified by dot-blotting in Subheading 3.3.4.

1. To obtain sufficient material for non-radioactive sequencing, repeat the PCR from Subheading 3.3.5. with only the selected clones as the templates. These PCR samples are checked on a gel and then purified using the Qiaquick PCR purification kit. Determine the concentration of the samples by OD_{260}, which should be at least 25 ng / µl.
2. Perform the sequencing reactions using 8 µl rhodamine dye terminator kit, 2 µl 1.6 µM primer SP6R or T7E, 250 ng ds DNA PCR product, and H_2O to 20 µl. Submit the obtained sequences to BLAST searches.

3.4. Further characterization of candidate genes

It is advisable to reconfirm the expression pattern for these genes which showed altered expression in the microarray experiments by an independent method, such as Northern blot analysis. In the case of less abundant genes, quantitative RT-PCR might be required.

Further analysis of interesting clones will be dependent on the nature of the gene. To further obtain information on the Rac-induced genes, we started to explore the effects of various growth factors and drugs on the transcription of these genes. We used reagents known to activate or to interfere with previously characterized signaling pathways of Rac. For example, the transcription levels of candidate genes can be compared between resting cells and cells stimulated with PDGF or EGF. Furthermore, Rac is known to activate e.g. the p38 MAP kinases and a drug, SB202190, is known as a selective inhibitor of these kinases [18]. The transcription of candidate genes can be compared between cells induced for Rac and either left untreated or treated with SB202190. These experiments are also performed using the microarray technique, allowing to monitor simultaneously the effect of these reagents on the genes identified by cDNA-RDA.

4. NOTES

1. A major challenge in cDNA-RDA experiments is the high risk of contamination, since cDNA-RDA is able to amplify very small differences between two representations. Hence, while performing cDNA-RDA, the use of gloves, sterile filter tips, single use individually wrapped pipettes, and sterile single use plasticware is highly recommended. Be aware that not all types of plastic withstand the phenol/chloroform solutions used. Therefore, use rather polypropylene than polystyrene tubes. Also, all reagents are used only for cDNA-RDA.
2. To prevent losing sequences later during preparation of the representations (Subheading 3.2.1.), one can design specific primers for the preparation of cDNAs. Short or unusually composed mRNA sequences might contain either none or only one *Dpn*II site. To avoid their loss, use primers with a *Dpn*II site added at the 5' end instead of standard random or oligodT primers.

Using large amounts of cells derived from an inducible expression system might not be possible in all cases. When starting with smaller amounts of cells (10^6), the Oligotex kit (Qiagen) is recommended for mRNA isolation. If working with even smaller amounts of defined starting material or tissue samples, it may be necessary to use specialized methods during the preparation of representations, such as discussed elsewhere [12, 6, 19]. When the samples to be used as tester or driver need to be pooled (e.g. progenitor cells of healthy individuals), it is advisable not to pool the cells or the mRNAs, but to prepare in parallel a representation from each individually and then to combine them.

3. Incubate in an incubator rather than in a waterbath or a thermoblock. An incubator is preferred as such to avoid evaporation of the solution and recondensation on the inner surface of the lid.
4. Be careful not to lose minute amounts of dried DNA in handling. This is a major source of low yields in the following PCR amplification steps. It is also important not to over-dry the DNA pellets, since they become more difficult to redissolve.
5. It is essential to reproducibly obtain representations before starting cDNA-RDA. If a typical yield of ca. 150 µg from 0.4 µg template is not obtained, this step has to be optimized (such as by preparing new buffers, new cDNAs and / or mRNAs).
6. Depending on the level of induction, the induced gene itself may become a major part of the difference product, when the induced sample is employed as the tester to identify upregulated genes. This can be suppressed by adding this particular transcript back to the driver during both rounds of cDNA-RDA. While others have recommended to substitute up to 50 % of the driver by the gene [6], we would advise to be more cautious and rather to substitute ca. 10 % of the driver. An optimal ratio can also be determined experimentally by titration. The Southern blot analysis described in Subheading 3.3.1. might not be quantitative enough to detect whether the addition had an effect on the composition of the DP2. However, the difference should be

notable in the percentage of individual clones identified by dot-blotting (in Subheading 3.3.4.) as being the induced gene.

7. There are only a few ways to judge the quality of the cDNA-RDA experiment during the procedure. It is essential, however, that the yields and amplification rates described throughout the protocol are achieved. Also, digests should result in downward shifts of band patterns in agarose gels (as in Subheading 3.2.1.4., step 3). Finally, equal aliquots of driver and tester representations, DP1, and DP2 can be run together on a gel (to perform the Southern blot analysis described in Subheading 3.3.1.). The observation of appearing or disappearing bands from representation to DP1 and DP2 may indicate that the cDNA-RDA has worked. However, a constant band pattern does not necessarily indicate that the cDNA-RDA has failed. Best is to proceed with the Southern Blot analysis and use its outcome as described in Subheading 3.3.1. to judge the quality of the cDNA-RDA experiment.
8. We found that inclusion of 12 % DMSO in this PCR buffer significantly increased the yield, while addition of Triton X-100 had no effect on the yield and reduced the clarity of the run on an agarose gel.

Acknowledgments

The authors would like to thank Joan Alexander for sequencing and Chet Cunha (Friend's Academy H.S., Glen Cove, NY) for technical assistance.

This research was supported by grants from the U.S. Army and the NIH to Linda Van Aelst, who is also a Kimmel Foundation and Foundation V award recipient. Arndt A. P. Schmitz is supported by a post-doctoral fellowship from the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany).

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FIGURE LEGENDS

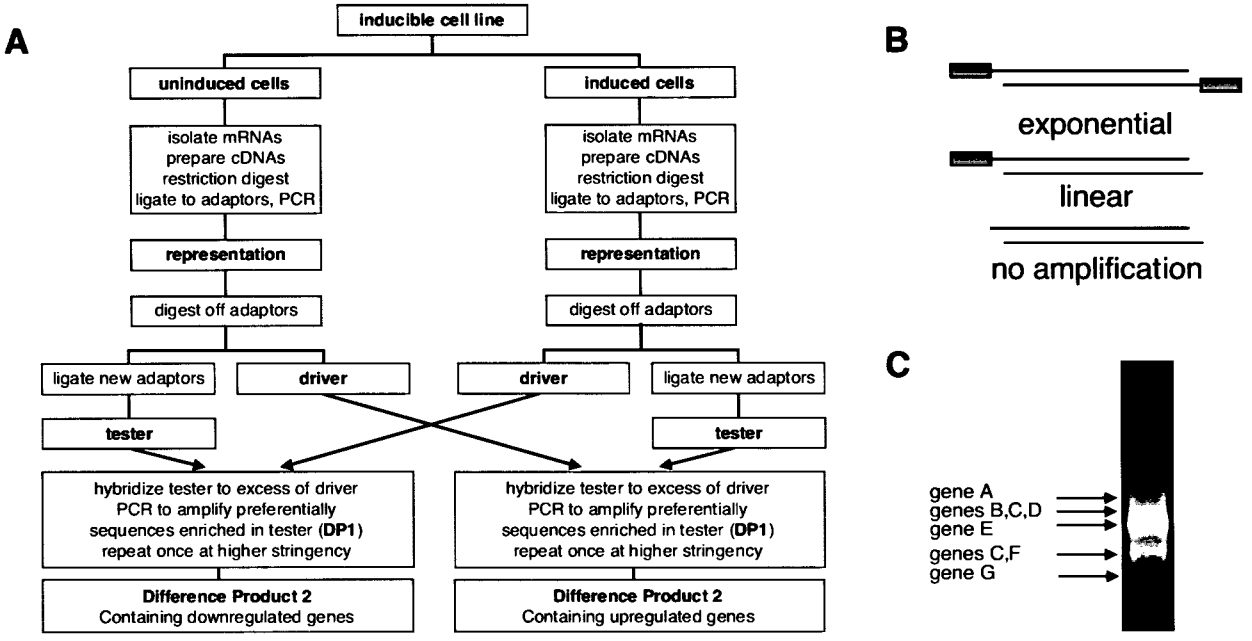
Fig. 1: Principle of cDNA-RDA. A. Flowchart of cDNA-RDA. A. From cells either induced or left uninduced for expression of the gene of interest, representations are prepared by isolation of mRNAs, synthesis of cDNAs, restriction digest with *Dpn*II, ligation to adaptors, and amplification by PCR. The adaptors are subsequently removed by digestion in order to use the representation as the driver in a cDNA-RDA experiment. To use the representation as the tester, the adaptors are cut off and replaced by newly ligated adaptors of different sequence. Then, the tester from one sample is hybridized to an excess of driver from the other sample and sequences enriched in the tester are selectively amplified by PCR to obtain the difference product one (DP1). Finally, the DP1 is used as the tester in a new round of hybridization and amplification to result in difference product two (DP2). Note that by performing two sets of reactions in parallel, using cells from each of the two samples once as tester and once as driver against the other sample, up- as well as down-regulated genes can be identified. **B.** The three possible outcomes of the tester/driver hybridization. If a sequence is unique to the tester or present at a higher molar ratio in the tester than in the driver, it will be exponentially amplified. If a sequence is found in both driver and tester to equal amounts, only the strand from the tester population bears the adaptor and the sequence will be linearly amplified. If the sequence is found only in the driver, neither strand contains the adaptor sequence and the sequence will not be amplified. **C.** A typical difference product after two rounds of cDNA-RDA visualized by agarose gel electrophoresis. A difference product consists of a series of visible bands superimposed on a 'smear'. As indicated, each band may contain fragments of several genes whose different sizes can not be resolved on an agarose gel. Furthermore, candidate genes may be contained in the 'smear'. Finally, different fragments of the same gene can occur at different positions in the gel, since cDNA-RDA is based on digested cDNA.

Fig. 2: Analysis of cDNA-RDA products by the microarray technique. 190 clones derived from a cDNA-RDA experiment using cells induced for RacV12 expression as the tester and uninduced cells as the driver were arrayed in duplicate. The array was then hybridized simultaneously to a representation from RacV12 induced cells (labeled in green) and to a representation from uninduced cells (labeled in red). The clones with different shades of green represent genes whose expression is upregulated as a result of RacV12 expression, whereas 'yellow clones' are transcripts unaffected by RacV12 expression. Genes downregulated by RacV12 would be identified as red spots, but are absent as expected from the design of this particular experiment. Such, microarraying allows rapid screening of a difference product for the clones with the highest differential expression ratio which are then further pursued.

Table 1: Sequences of the used Primers.

Name	Sequence	Use	Ref.
RBgl24	AGCACTCTCCAGCCTCTCACCGCA	Representation	11
RBgl12	GATCTGCGGTGA	Representation	11
NBgl24	AGGCAACTGTGCTATCCGAGGGAA	1st round of cDNA-RDA	11
NBgl12	GATCTTCCCTCG	1st round of cDNA-RDA	11
IBgl24	TCAGCATCGAGACTGAACGCAGCA	2nd round of cDNA-RDA	a)
IBgl12	GATCTGCTGCGT	2nd round of cDNA-RDA	a)
SP6R	GGTGACACTATAGAATACTCAAGC	Single colony PCR	b)
T7E	TGTAATACGACTCACTATAGGGC	Single colony PCR	b)
Random nonamer	NNNNNNNNN	Probe labeling	a)

Footnotes to Table 1: a) Robert Lucito, unpublished results; b) this report.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hybridize tester to excess of driver
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sequences enriched in tester (DP1)
repeat once at higher stringency
hybridize tester to excess of driver
PCR to amplify preferentially
sequences enriched in tester (DP1)
repeat once at higher stringency
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Containing upregulated genes
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genes B,C,D
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genes C,F
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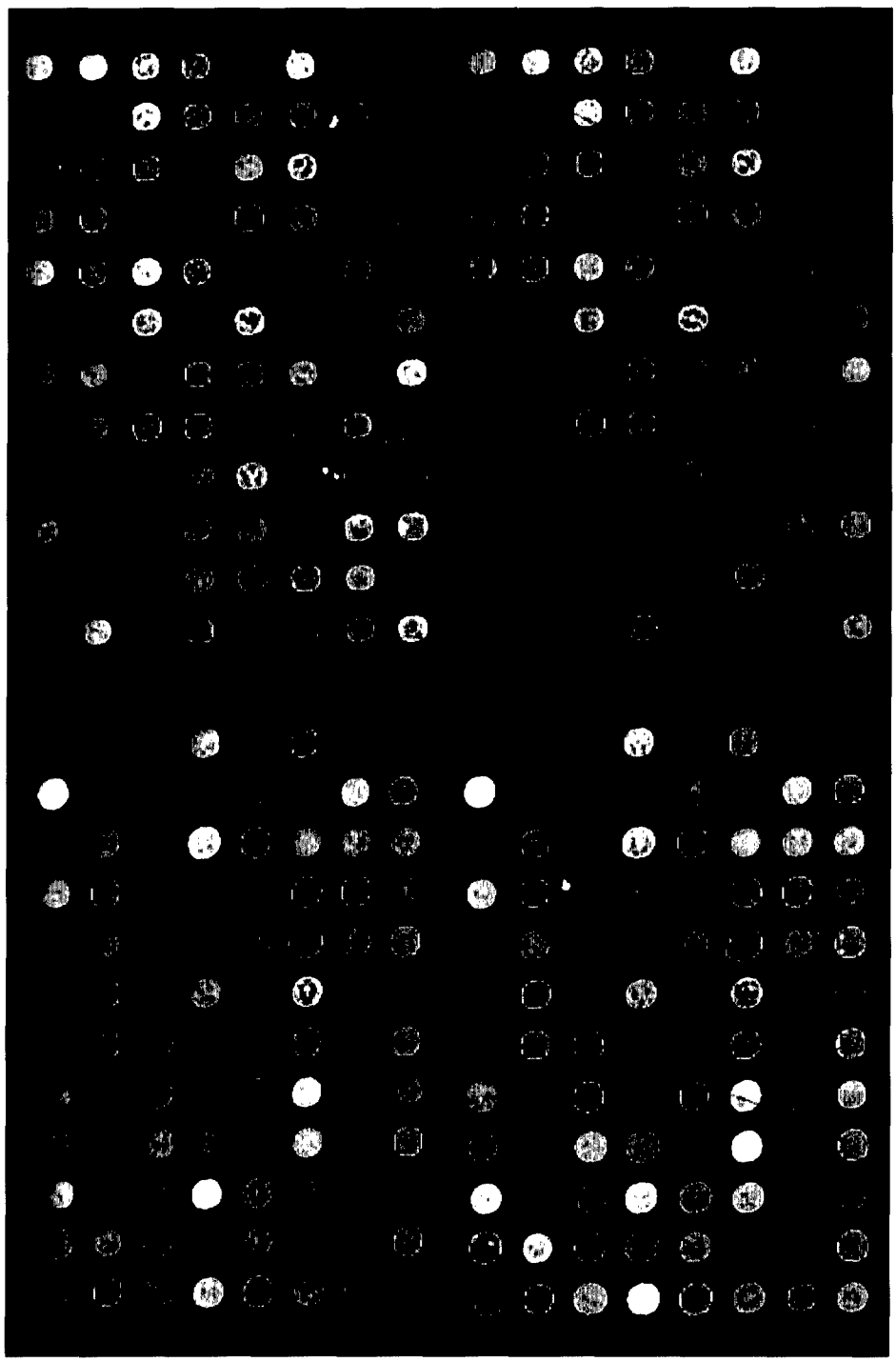

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